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(54) Title: PROBE

(57) Abstract: A probe comprising an optionally porous, polymer bead with at least one type of nanocrystals associated therewith, said bead and nanocrystals being coated with a polymeric coating.

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# Probe

This invention relates to a process for the preparation of synthetic beads comprising nanocrystals, in particular to a process for the preparation of beads comprising a combination of nanocrystals as well as to the resulting probes themselves and the use thereof as probes in biological applications.

A wide variety of chemical and biological assays are known for identifying an analyte of interest in a given sample. For example, immunoassays such as enzyme-linked immunosorbent assays (ELISA) are used in numerous diagnostic, research and screening applications.

Assays generally utilise detectable labels to identify the analyte of interest. Hence, radiolabelled molecules and compounds are frequently used to detect biological compounds both *in vivo* and *in vitro*. However, due to the inherent problems associated with the use of radioactive isotopes, non radioactive methods of detecting biological and chemical compounds are preferable.

Thus, fluorescent labelling of biological systems has become a widely used and known analytical tool in modern biotechnology. Applications for such fluorescent labelling include technologies such as medical fluorescence microscopy, histology, flow cytometry, DNA sequencing, immunoassays, binding assays, separation etc. Fluorescent molecules are therefore commonly used as tags for detecting an analyte of interest.

Conventionally, fluorescent labelling involves the use of an organic dye molecule bonded to a moiety which selectively bonds to a particular biological system, the presence of which is then identified by excitation of the dye molecule to cause it to fluoresce. A great number of appropriate dyes are known, however there remain a number of limitations with the use of such

compounds.

Firstly, the emission of light of visible wavelengths from an excited dye molecule is usually characterised by a broad emission spectrum as well as a broad tail of emissions on the red side of the visible electromagnetic spectrum. As a result, there is a severe limitation on the number of different organic dyes which may be used simultaneously or sequentially since it is difficult to discriminate between the detectable substances.

Moreover, the majority of fluorescent dyes have a relatively narrow absorption spectrum therefore requiring multiple excitation or a broad spectrum excitation source which would not be specific for a particular dye. The narrow absorption spectrum therefore limits usefulness of organic dyes.

A yet further limitation is that the fluorescence exhibited by a compound may deteriorate upon prolonged and/or repeated exposure to light and conversion of the dye or decomposition of the dye into non-fluorescent species may occur and may be irreversible. Decomposition products may also interfere with separation, analysis or binding procedures.

A final limitation is that certain lower molecular weight dyes in everyday usage may not provide a bright enough signal for ready detection.

In order to overcome many of the problems associated with fluorescent materials, semiconducting nanocrystalline particles may be employed and such particles have been shown to be reliable as detectable labels in a variety of biological systems. These nanocrystals are capable of producing luminescence and/or absorption when excited by an electromagnetic source and may be tailored so as to have characteristic spectral emissions. The emissions are of high intensity, narrow linewidth (hence there is much less chance for spectral overlap) and excitation of the

nanocrystals can be achieved using electromagnetic radiation in the UV or visible range.

In US 5,990,479 an organoluminescent semiconductor probe is suggested formed from a nanocrystal compound linked to an affinity ligand, optionally via a linking group. The affinity ligand is selected for its ability to bind a desired detectable substance in a sample.

WO 00/68692 describes the use of semi-conducting nanocrystals for detecting one or more target analytes in a sample. The various methods described involve the use of semi-conductor nanocrystal conjugate, i.e. the nanocrystal is linked to a specific binding molecule, which directly or indirectly attaches to the analyte of interest. It is also suggested that the nanocrystal may be made part of a solid support, e.g. by binding the nanocrystal to a styrene or other polymer monomer and polymerising this monomer with others to form, for example, a polystyrene bead.

The present inventors have surprisingly found that nanocrystal particles can be employed much more readily in an assay procedure if the particles are associated with a polymer bead, e.g. contained within pores present in a polymer bead or deposited on the surface of a preformed bead. Such beads are preferably preswollen, optionally porous beads which can be made using the Ugelstad two-step swelling technique. Such nanocrystal containing beads, from hereon referred to as probes, can be readily prepared and utilised in a diverse range of assays. Moreover, since by use of differing combinations of nanocrystals the emission spectra of the probe may be tailored, probes can be provided with characteristic "bar-coded" emissions.

Thus, viewed from one aspect the invention provides a process for the preparation of a luminescent probe comprising mixing an optionally porous, polymer bead with at least one type, preferably at least two types, of nanocrystal so that the nanocrystals associate with,

e.g. enter the pores of or deposit onto, said bead; and subsequently coating said bead to form said probe.

Viewed from another aspect the invention provides a probe comprising an optionally porous, polymer bead with at least one type of nanocrystals associated therewith, said bead and nanocrystals being coated with a polymeric coating.

Viewed from a further aspect the invention provides the use of a probe as hereinbefore described in an assay.

Viewed from yet another aspect the invention provides a method for detecting an analyte in a sample comprising mixing said sample with a probe as hereinbefore described said probe carrying at least one affinity ligand; allowing the analyte to bind to said probe; and detecting the resulting probe-analyte conjugate, e.g. spectroscopically.

A nanocrystal (also referred to as a colour particle) as used herein is an inorganic single crystal particle between approximately 1 and 100 nanometres in diameter. The nanocrystals of use in the probes of the invention must be luminescent, i.e. they must be capable of emitting electromagnetic radiation upon excitation.

The nanocrystals used in the invention will be described further below and are commercially available under the Trade Names Biocrystals and Quantum Dots (Qdots). The preparation of the nanocrystals is also described in, amongst others, US 5,251,018, US 5,262,357 and US 5,505,928 which are herein incorporated by reference.

The nanocrystals may have any suitable diameter although the nanocrystals should be small enough to be able to enter into the pores of the bead if these are present. Preferably, nanocrystals should be between 2 and 50 nanometres, especially 2 to 20 nanometres, especially 4 to 10 nanometres in diameter. The nanocrystals should however, have a diameter greater

than 0.5 nm.

The nanocrystals are capable of emitting electromagnetic radiation upon excitation and are therefore luminescent. Typically, they are spherical in nature and are preferably monodisperse, i.e. all the nanocrystals are within a defined particle size range. The nanocrystals may include a core of one or more first semi-conductor materials which may be surrounded by a shell of a second semi-conducting material. Such a species is referred to as a core/shell nanocrystal and again such species are well-known in the art.

The surrounding shell material should preferably have a band gap energy that is larger than the band gap energy of the core material and may be chosen to have an atomic spacing close to that of the core substrate.

The core and/or shell is a semi-conductor material and generally comprise elements in groups 11, 12 and II-VI of the periodic table. Particularly preferably, an element from group IIB and VIB, IIIB and VB, or IVB and IVB form the core. Thus, suitable materials for the manufacture of the nanocrystals of use in the invention include the sulfides, selenides and tellurides of zinc, cadmium, mercury, magnesium, calcium, strontium, barium. Also of use are GaN, GaP, GaAs, GaSb, InN, InP, InAs, InSb, compounds of germanium, silicon and certain alloys.

Preferably, the nanocrystal core is made from CdS, ZnS, CdSe, CdTe, ZnSe, ZnTe, GaP or GaAs.

The shell substance, if present, is preferably a semiconductor material as well and may therefore be selected from those listed above.

The nanocrystals will preferably emit light with a narrow linewidth, e.g. a band of 40 nm or less, preferably 20 nm or less thus permitting the simultaneous use of a plurality of differently coloured organoluminescent nanocrystal species without emission overlap.

The nanocrystal may also be coated with an organic capping agent as is well known in the art. The capping agent should have an affinity for the nanocrystal surface and may therefore be, for example an monomer/polymer species. The capping agent may also be present to aid association of the nanocrystal with the optionally porous bead, for example by allowing interaction of the capping agent with a suitable group present on the surface of the bead or within a pore in the bead. Thus for example, a nanocrystal carrying a carboxyl capping agent may deposit readily onto the surface of a bead carrying an amine functionality on its surface. Alternatively, the capping agent may be hydrophobic so as to allow ready diffusion of a nanocrystal into the pore of a bead which has been prefunctionalised so as to provide a similar hydrophobic environment. Preferably, the nanocrystals comprise a hydrophobic capping agent, e.g. a capping agent of formula $O=P(C_{6-12}\text{-alkyl})_3$ such as $O=P(C_8\text{-alkyl})_3$ or $P(C_8\text{-alkyl})_3$ or mixtures thereof.

A comprehensive discussion of nanocrystals can also be found in WO00/68692 which is herein incorporated by reference.

Whilst the probes of the invention may comprise only one type of nanocrystals (e.g. CdSe nanocrystals of a particular size and emission spectrum) it is preferred if at least two different types of nanocrystal are employed thus allowing the preparation of probes having an emission spectrum which can act as a kind of "bar code". By using different combinations of nanocrystals, e.g. different sizes, size distributions, chemical composition etc each probe can be made to have a very characteristic emission spectrum, i.e. a particular linewidth, intensity, luminescence lifetime etc. Hence, a probe comprising two types of nanocrystals can be said to exhibit at least two distinguishable emissions in its spectrum. When a number of such barcoded probes are

used together in an assay, it is easy to determine which probe has bound to which analyte simply by recording the nature of the probe's emission spectrum. The technique therefore allows valuable information to be ascertained. Moreover, the probes may also be used to label biological entities such as chromosomes for spectral karyotyping or the like.

The polymer beads in which the nanocrystals are to be held may be prepared using techniques known in the art, e.g. core/shell beads, but are preferably prepared using the well-known Ugelstad two-step swelling process and the improvements thereon described comprehensively in WO 00/61647. Beads made in this way are, from hereon, called Ugelstad beads.

Before the advances in bead preparation made by Professor Ugelstad, polymer beads were produced by diffusing a monomer and a polymerisation initiator into polymer seeds in an aqueous dispersion. The seeds swell and following initiation of polymerisation, large polymer particles are produced. The maximum volume increase in such a process is typically x5. Professor Ugelstad found that the capacity of the seeds to swell could be increased to very high levels (e.g. x125) if an organic compound with relatively low molecular weight and low water solubility was also diffused into the seeds before the bulk of the monomer is used to swell the seeds. This is now achieved by using the polymerisation initiator itself as the low molecular weight and low water solubility compound.

Hence, a swellable latex seed particle made of any conventional polymer or oligomer, e.g. styrene oligomer, has diffused into it a polymerisation initiator and subsequently a monomer such as a styrene, acrylate, unsaturated chloride, ester, acetate, amide and alcohol monomer. Polymerisation is then initiated. In particular, the "swollen" beads (i.e. beads produced by swelling) of use in the invention may comprise

polystyrene (including high density polystyrene latexes such as brominated polystyrene), polymethylmethacrylate and other polyacrylic acids, polyacrylonitrile, polyacrylamide, polyacrolein, polydimethylsiloxane, polybutadiene, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidene chloride and polydivinylbenzene.

Preferably, the beads are made from combinations of styrenes, acrylates and/or methacrylates. More preferably the beads are made from styrene, divinylbenzene, acrylates and methacrylates, especially styrene and divinylbenzene.

Typically, the resulting beads are spherical and monodisperse. This is important since agglomeration of beads may cause signal detection problems. If non-monodisperse particles are employed in the probes of the invention agglomeration may occur and it will then be impossible to tell whether an intense signal is due to a particular probe or simply due to the large number of probes which agglomerate. Light intensity measurements may therefore be of little value. If monodisperse beads are employed then signal strength from the probes of the invention will be dependent on intensity of the nanocrystals incorporated in each probe allowing an accurate concentration dependent analysis to be made. This is an important advantage in a multiplex assay.

Beads may be non-porous (compact) or porous. To make a bead porous, a porogen is employed as is known in the art. The porogen is typically added prior to monomer polymerisation. Suitable porogens are low molecular weight aliphatic or aromatic hydrocarbons or alcohols such as heptane, toluene or cyclohexanol. The use of glycidyl methacrylate in the final swelling stage of the Ugelstad process has been found to yield beads which are more readily functionalised then conventional beads and this forms a further aspect of the invention.

Beads may also be cross-linked as is known in the art.

The resulting beads may have a diameter of between 0.5 and 100 microns, e.g. 2 to 20 microns, especially 2 to 5 microns. If pores are required, a pore volume of 20 to 60% is preferable. The pores are generally between 5 and 200 nanometres in diameter and may be up to 200 nm in depth.

Once the optionally porous beads have been prepared the nanocrystals need to become associated with the beads. By this is meant that the nanocrystals need to bind chemically, physically or electrostatically with the bead or simply become associated with the bead via diffusion or suction into the pores of the bead. Preferably, the nanocrystals associate physically or electrostatically with the beads, e.g. via deposition onto the bead surfaces. This may be achieved using any convenient process.

In order to ensure association of the nanocrystals with the beads it may be necessary to functionalise the beads before contact between the nanocrystals and the beads is made. For example, whilst the surface of the beads of the invention may be left unfunctionalised, it is preferred if the surfaces are coated with appropriate chemical functionalities to ensure that the nanocrystals are in a suitable chemical environment on the bead surface or within the pores. Hence, the surface of the bead/pore may be coated with hydrophobic/hydrophilic groups or may be functionalised so as to be charged depending on the nature of the nanocrystal to be added.

Clearly, the surfaces of the bead and nanocrystal unit are designed so as to readily associate, i.e. if the pore surface comprises amine functionalities then the nanocrystal may comprise a corresponding acid functionality so that binding or deposition can occur. Similarly, a positive charge in the pore surface needs to be associated with a negative charge on the nanocrystal. Functionalisation/coating of the bead

surface is readily achieved by known processes.

The beads may therefore be aminated so as to allow the binding of a carboxy functionalised nanocrystal. Alternatively, the beads may be functionalised to allow hydrophobically functionalised nanocrystals to become associated with the beads. Nanocrystals may also diffuse into the pores of the beads if a suitable concentration gradient exists between the pores and the medium in which the nanocrystals are in. Nanocrystals may also displace groups present on the bead or pore surfaces. The skilled artisan would readily devise methods for incorporating nanocrystals into the porous beads.

An increased absorption of nanocrystals is seen if the bead coating contains functional groups which are known to coordinate with the shell of the nanocrystal, e.g. amines, thiols, phosphines, phosphine oxides, pyridine, furans etc.

In one embodiment of the invention, the polymer bead is coated with an epoxide polymer and nanocrystals having a capping agent of formula $O=P(C_{6-12}\text{-alkyl})_3$ are associated therewith.

The introduction of the nanocrystals into or onto the beads should take place in a medium that favours association of the nanocrystals with the beads. The nature of the medium clearly depends on the nature of the nanocrystal as well as the nature of the pore surface but where the nanocrytals carries a polar functionality, an alcohol such as ethanol is a convenient medium. If the nanocrystal carries a non-polar functionality suitable solvents for the absorption process include toluene, 1,4-dioxane, butyl acetate, butanol, propanol, chloroform and mixtures thereof.

In one embodiment of the invention, the nanocrystals are found within the pores of a porous bead, e.g. at least 50%, preferably 80%, especially 100%, of the nanocrystals associated with the bead are

to be found in a pore. Alternatively, the nanocrystals are deposited on the surface of a non-porous bead, e.g. by specific deposition.

The amount of nanocrystals required per bead will be readily determined by the person skilled in the art and will depend on the nature of the nanocrystal, bead and the intensity of signal required. Typically, however, to prepare 10 g of probes according to the invention 1 ml to 100 ml of 2.5 $\mu$M nanocrystal dispersion is added. The association procedure may take from 10 minutes to 24 hours, e.g. 2 hours.

Ugelstad beads are often made magnetisable by adding a magnetisable material (e.g. iron material) to the porous beads. This is of course possible in the present invention where the magnetisable material can be added before, simultaneously with or after addition of the nanocrytals to the beads. For example, iron (II) sulphate or iron (II) oxide particles are incorporated into beads when contact between the beads and the iron compound is made. Preferably the beads can be nitrated to allow the association with the magnetisable particles. This chemistry is well-known and will be readily achieved by the person skilled in the art. By this technique magnetisable, luminescent probes can be prepared and these form a yet further aspect of invention.

Preferably, the magnetisable particles are introduced into the pores of a bead prior to association of the beads with the nanocrystals. Whilst the nanocrystals may then be additionally added to the pores or deposited on the bead surfaces, preferably the bead is then coated to ensure that the magnetisable particles do not dissociate from the beads whilst also functionalising the bead surface to allow nanocrystal association. Coating may be achieved using an epoxide polymer for example. Nanocrystals can then be associated with the beads as described in detail above

to form the probes of the invention.

Since the nanocrystals associated with the probes of the invention may be "free floating" within the mesh of the bead or deposited on the surface of a bead, it is necessary to coat the probes with a suitable coating material so as to prevent loss of nanocrystals in the reaction medium or sample and to prevent possible degradation of the nanocrystals by chemicals, buffers etc. The use of a coating has proved vital since in the absence of a coating nanocrystals can be lost or degraded by chemicals, buffers etc which the probes contact. By using a coating, the probes are greatly stabilised giving the probes of the invention a longer shelf-life and a reproducible and constant emission allowing a higher degree of multiplexing.

Hence, after the nanocrystals have been associated with the beads, the resulting probes are coated using, for example, a polymer again using known processes. Typically the polymer used as for the external coat is an epoxy or polyurethane polymer or a glycidyl ether. Alternatively, probe coating can be achieved by cross-linking a coating already present from an earlier stage of the probe synthesis. For example, where a epoxide polymer has been used to coat the beads after the introduction of magnetisable particles into bead pores, this polymer can be crosslinked with, for example, a diisocyanate to produce an external polymer coating.

Hence, viewed from a further aspect the invention provides process for the preparation of a luminescent probe comprising:

(I) mixing a porous bead with magnetisable material so as to allow magnetisable material to enter the pores of said bead;

(II) coating said magnetisable bead with a polymer coating, said coating optionally comprising a

functional group that coordinates with the surface of a nanocrystal;

(III) contacting the coated magnetisable bead with at least one type of nanocrystal so that said nanocrystals associate with said bead;

(IV) optionally coating the resulting probe by cross-linking said polymer coating.

After this coating procedure has taken place the surface chemistry of the bead may be manipulated to provide epoxy, hydroxy, amino, etc. functionalities on the surface to allow binding to affinity ligands. In a preferred embodiment the result of the secondary coating procedure is a bead surface already suitable to bind to affinity ligands, e.g. by using prefunctionalised polymer monomers in the manufacture of the secondary polymer coat. If this cannot be achieved functionalisation of the bead surface is readily achieved as is known in the art, e.g. by reaction with certain bifunctional reagents.

The probe can then be bound to an affinity ligand the nature of which will be selected based on its affinity for the particular detectable substance whose presence or absence in a sample is to be ascertained. The affinity molecule may therefore comprise any molecule capable of being linked to a luminescent probe which is also capable of specific recognition of a particular detectable substance. Affinity ligands therefore include monoclonal antibodies, polyclonal antibodies, antibody fragments, nucleic acids, oligonucleotides, proteins, polysaccharides, sugars, peptides, peptide nucleic acid molecules, antigens, drugs and ligands. Lists of suitable affinity ligands are available in the published literature and are universally well known. In general, affinity ligands used previously in assays involving fluorescent dyes are

of use in the present invention. The use of further binding partners, secondary affinity ligands and linking groups which is routine in the art will not be discussed further herein although it will be appreciated that the use of such species with the beads of the invention is possible if desired.

The nature of the external substance on the bead is selected for the target material to be labelled and optionally detected. For example, nucleic acid detection generally involves probing a sample thought to contain target nucleic acids using a nucleic acid probe that contains a nucleic acid sequence that specifically recognises, e.g. hybridises with, the sequence of the target nucleic acids, such that the nucleic acid affinity ligand and the target nucleic acids in combination create a hybridisation layer.

The target material is optionally a material of biological or synthetic origin but is present as a molecule or as a group of molecules, including, antibodies, amino acids, proteins, peptides, polypeptides, enzymes, enzyme substrates, hormones, lymphokines, metabolites, antigens, haptens, lectins, avidin, streptavidin, toxins, poisons, environmental pollutants, carbohydrates, oligosaccharides, polysaccharides, glycoproteins, glycolipids, nucleotides, oligonucleotides, nucleic acids and derivatised nucleic acids, DNA, RNA, natural or synthetic drugs, receptors, virus particles, bacterial particles virus components, cells, cellular components, natural or synthetic lipid vesicles, polymer membranes, polymer services and particles and glass and plastic surfaces.

The nanocrystals in the probes of the invention may be excited by conventional means. The nanocrystals are preferably excitable over a broad bandwidth but exhibit emission only in a narrow waveband, i.e. in sharp contrast to organic dyes. Thus electromagnetic

radiation of wavelength ranging from X-ray to ultraviolet to visible to infrared may be used to excite the luminescence in the nanocrystals.

The nanocrytals may also be excited by bombardment by a particle beam, e.g. electron beam. Advantageously, one excitation source can be employed to excite several different nanocrystal types, i.e. several nanocrystal types which give off radiation at different frequencies thus permitting simultaneous excitation and detection of the presence of several nanocrystal types and hence several detectable substances from a sample.

Thus, for example, a laser source of a given frequency, e.g. blue light, may excite a nanocrystal which emits green light and a further nanocrystal which emits red light. Detection of both red and green light would show the presence of the biological target to which the bead in which the nanocrystal resides was designed to bind.

Detection of the emission spectra of the excited nanocrystals can be achieved using a commercially available detection system such as a spectrometer. Detection of a particular nanocrystal emission will of course signify detection of the analyte to which the polymer bead/nanocrystal system was designed to bind.

The probes of the present invention may be employed in a wide variety of assays. Perhaps in its simplest form probes with a suitable affinity ligand, e.g. an antibody molecule or a general or specific affinity ligand, can be added to a biological sample, e.g. a crude blood or cell lysate, containing a target protein. After the probe has bound to the target protein a specific detecting antibody may be added to bind to the probe/target protein conjugate. The conjugate may then be separated from the remaining sample, conveniently magnetically, and then bound to a suitably functionalised solid substrate, e.g. a streptavidin functionalised substrate via the specific detecting

ligand, e.g. a biotinylated antibody, thereby allowing detection of the probes, and hence the target protein, by their luminescence.

In a similar fashion, a microorganism or a specific type of cell could be isolated from a sample, e.g. a crude blood or tissue sample, by attaching to the microorganism or cell the magnetisable probes of the invention via a suitable affinity ligand. After magnetic separation of the microorganism/cell:probe conjugate a detecting ligand, e.g. an antibody, may be bound to the conjugate allowing the conjugate to be coated onto a solid substrate, such as a streptavidin coated chip or sensor. Once bound, the type of probes present can be assessed using the nanocrystal emission spectra and hence detection of particular microorganisms or cells may be effected.

Hence, viewed from a further aspect the invention provides a method for detecting a target in a sample comprising mixing a probe with a suitable affinity ligand with a biological sample, e.g. crude blood or cell lysate, containing said target;

allowing the probe to bind to the target to form a probe:target conjugate;

mixing said conjugate with a specific detecting ligand;

separating the conjugate from the sample, e.g. magnetically;

binding the conjugate to a solid substrate via said specific detecting ligand and detecting the probe.

Said analyte can of course be a protein, cell, microorganism, etc.

A major benefit of the probes of the present invention is the ability to use more than one type of probe in one assay procedure. Hence, a mixture of different probes, i.e. probes with different spectral emissions, comprising affinity ligands for a variety of

targets, may be added to a crude sample whereupon various components of the crude sample can be bound and then separated using the techniques described above. After coating onto a solid substrate, e.g. a sensor, probe detection takes place and due to the different emission spectra of each different type of probe, many analytes can be detected from a single sample. For example, particular proteins within a crude sample or particular cells within a sample may be detected.

It is, of course, also possible to utilise the probes of the invention to detect the presence of a specific nucleic acid. Here, the probes simply need to carry a suitable capture oligonucleotide to bind to a nucleic acid of interest. The resulting conjugate can then be labelled e.g. using a standard polymerase/ biotinylated nucleotide procedure or by hybridisation with a specific biotinylated or other labelled oligonucleotide. After heating/washing (and hence removal of the nucleic acid detected) and magnetic separation, probes can be bound to a solid substrate and those carrying the biotin detected. When the nucleic acid analysed is mRNA then such assays will allow the detection of particular genes which are expressed in the sample of interest.

Hence, viewed from a still further aspect the invention provides a method for detecting a plurality of targets in a sample comprising mixing a plurality of different types of probes, each type of probe having a different affinity ligand, to a biological sample;

allowing the probes to bind to a plurality of targets within the sample to form probe:target conjugates;

mixing said conjugates with a specific detecting ligand;

separating the conjugates from the sample, e.g. magnetically;

binding the conjugates to a solid substrate via

said specific detecting ligand and detecting the probes via their different emission spectra.

Where the target is nucleic acid the affinity ligand is preferably an oligonucleotide and the specific detecting ligand is a label such as a biotinylated or other labelled oligonucleotide.

A more sophisticated assay allows the detection of particular genes which may be expressed in a cell or tissue or indeed any other appropriate biological entity, e.g. a microorganism. In this procedure, standard Dynabeads® are used to isolate a cell, microorganism etc from a sample of interest. The isolated cell/bead conjugate is then placed in lysis buffer, the cells lysed and the beads, cell membranes and other cell debris removed. Other methods of isolating mRNA may be used. The remaining supernatant containing mRNA (e.g. from $10^4$ cells) is transferred into a medium comprising a variety of probes of the invention with varying gene-specific oligonucleotide affinity ligands or capture oligonucleotides, e.g. 20 different types. The mRNA binds to certain of the probes having suitable affinity ligands or capture oligonucleotides and after washing to remove unbound mRNA and magnetic separation a label can be attached to those probes which are bound to mRNA. For example, a suitable label for use in the detection of mRNA is a labelled dT oligonucleotide. Alternatively, labelling may be achieved by primer extension, incorporating labelled nucleotides, optionally of the di-deoxy type or primer extension to incorporate a biotinylated nucleotide followed by addition of streptavidin with a strong fluorescent label.

The probes can then be transferred to a solid substrate and it is then possible to detect which probes are bound to a label and hence what mRNA sequences (and therefore genes) are present. Detection is conveniently achieved by microscope image analysis and the use of an

IR laser. For example, a first laser may be employed to quantify the bound mRNA. A blue laser may then be employed to excite the nanocrystals allowing detection of which nanocrystals are bound to the target.

This more sophisticated assay is also suitable for detecting proteins from specific cells, microorganisms etc or detecting other forms of nucleic acid, e.g. DNA. Thus, a variety of different probes are employed and using techniques as described above, it is possible to identify which probes have bound protein, DNA etc and hence which proteins, DNA etc are present.

Hence, viewed from a still further aspect the invention provides a method for detecting a plurality of nucleic acid sequences in a sample comprising mixing a plurality of different types of probes, each type of probe having a different gene-specific oligonucleotide affinity ligands, to a sample comprising free nucleic acid;

allowing the probes to bind to a plurality of free nucleic acid sequences within the sample to form probe:nucleic acid conjugates;

separating the conjugates from the sample, e.g. magnetically;

labelling said conjugates, e.g. with a dT oligonucleotide;

binding the conjugates to a solid substrate and detecting the labels/probes.

The assays described above can also be carried out using conventional, i.e. non-nanocrystal probes and this forms a further aspect of the invention. Moreover, the person skilled in the art will be able to devise other assays in which the probes of the invention may be utilised.

For example, the probes of the invention may be of use in single nucleotide polymorphism (SNP) analysis, e.g. genotyping of disease related SNP's. In a diagnostic application, a large number of SNP's will

need to be analysed to determine a patient's genotype, either to characterise a disease or to design personalised therapies. Depending on the situation, the relevant number of SNP's to be analysed from a single sample may be within the range of 10 to 2000. A flexible, multiplexed SNP scoring platform is therefore required and a probe based platform is an example.

Thus, genomic DNA for genetic typing of a patient may be isolated from any cell e.g. a white blood cell, using conventional techniques, e.g. using an automator as sold by Thermo Labsystems. SNP scoring may then be achieved using DNA sequencing, sequence specific oligonucleotide hybridisation (SSO) or sequence specific priming of a PCR reaction (SSP).

Hence, a suitable assay may have the following steps:

1. add multiple PCR primer sets, where one primer of each is biotinylated.
2. Run PCR
3. Isolate PCR products with Dynabead streptavidin.
4. Generate single strands
5. Add SSP primer with sequence tags, to single stranded templates.
6. Add DNA polymerase and fluorescent-labelled ddNTP's for +/- primer extension.
7. Wash, denature, remove any beads and templates.
8. Add and bind +/- labelled primers to probes with antitag oligonucleotide probes.
9. Wash, remove liquid and transfer to microscope slide.
10 Read labels and bead coding in fluorescent microscope.

Such an assay forms a still yet further aspect of the invention.

The probes of the invention may also have utility in microfluidic devices. The use of such devices for

the rapid detection of DNA, proteins, or other molecules associated with a particular disease is described in detail in WO01/94635 and WO02/29106. Detection of targets is achieved by measuring a signal from a detectable reporter, e.g. a fluorescent reporter. The probes of the present invention are suitable as detectable reporters for use in microfluidic devices and this forms a further aspect of the invention.

The invention will now be described further by reference to the following examples.

**Example 1**

**Monosized fluorescent coated particles**

**Acrylic monosized particles**

5.0 ml of dioctanoylperoxide, 42 ml of $H_2O$ and 0.15 g of sodium laurylsulphate is homogenised to an emulsion. This emulsion is combined with 23.1 ml of a latex consisting of monosized polystyrene latex having a diameter of 0.95 $\mu$m. The amount of latex added contains 2.5 ml of polystyrene particles and 20.6 ml of a solution of 0.15% SDS in water. After stirring for 24 hours a mixture of 50 ml glycidylmethacrylate, 70 ml of ethylene glycol dimethacrylate, 200 ml of cyclohexanol, 800 ml water and 10 g pluronic F68 is swelled for 2 hours and polymerized at 67 degrees Celsius for 10 hours.

The mixture is cooled and washed with water and methanol to remove the cyclohexanol. The process gives monosized particles.

**Amination**

To 10 gram of the above particles in 100 ml water is added 5 ml of aminoethanol. The suspension is heated to 60 degrees Celsius. After stirring for 6 hours the suspension is cooled and the particles are washed on a

filter with water.

**Staining**

To 10 gram of aminated particles in a 150 ml water suspension 5 ml of 5$\mu$M carboxylic acid modified CdSe nanocrystals with emission wavelengths 608 and 645 nm (Quantum Dot Corporation) in ethanol is added. After 2 hours stirring the particles are concentrated on a filter.

Flow cytometry shows that the particles are fluorescent, and emit light in two channels.

**Coating process**

5 gram of fluorescent particles are suspended in 50 ml of water. After heating to 50 degrees Celsius 3 gram of Araldit DY-026 is added dropwise over 6 hours. After cooling and washing the particles with water, the reaction yields 6.5 gram of coated monosized particles. Flow cytometry shows that the particles are fluorescent, and emit light in two channels.

**Example 2 - Coloured Monosized porous beads**

Porous epoxy-functional acrylic beads with a pore volume of 50% and a diameter of 8.2 $\mu$m were made as described in WO 00/68692 and US 4,336,173

Modification with 2,2 -(ethylenedioxy) diethyl amine

15 gram of the epoxy functional porous bead was dispersed in 255 gram butyl acetate. 30 gram of 2,2 -(ethylenedioxy)diethylamine was added to the dispersion. The mixture was heated to 70$^0$C and left for 2 hours at 70$^0$C with shaking. The dispersion was washed sequentially with a 50wt% mixture of butylacetate and methanol until all the excess of amine was removed (confirmed by ninhydrine-test). The beads were dispersed in toluene to

a final dry content of 10,5 wt%.

Adsorption of trioctylphosphine (TOP)/ triooctylphosphineoxide (TOPO) stabilized quantum dot (ZnS/CdSe) and coating of the amine modified beads

1 mL of a 2,5 $\mu$M dispersion of TOP/TOPO Qdot (Quantum Dot Corp.) with an emission maximum at 608 nm were flocculated by adding 1 mL methanol. The supernatant was removed and to the quantum dot precipitate was added 1mL toluene and a further 4 mL of a 1:1 mixture of toluene and 1,4 dioxane.

1 gram of amine-modified porous beads from above were dispersed in 16 mL of a 1 :1 mixture of toluene and 1,4 dioxane. The bead dispersion was transferred to a flask and the quantum dot dispersion was added drop wise while stirring. The mixture was stirred for 1 hour at room temperature.

Visual inspection with a UV-lamp confirmed that the beads were fluorescent.

30 mL butyldiglycidylether was added slowly to the mixture and heated to 60$^0$C. After 4 hours the mixture was cooled and transferred to a centrifugal tube. The resulting beads were washed with a 1:1 mixture of 1,4 dioxane and toluene. Further the beads were washed with methanol and water, and finally dispersed in water.

Fluorescence spectroscopy showed that the emission maximum of the colored bead dispersion was 608 nm.

The beads were analysed in a fluorescence microscope (Olympus BX61). The excitation source was a 100 W mercury lamp with a band pass excitation filter with transmission interval: 425- 475 nm, and a band pass

emission filter with transmission interval: 595 - 625 nm. The objective used was a 40x UplanApo. The pictures were taken with a F-view digital camera (4096 grey levels) and analysed with AnalySIS Pro (Soft Imaging system) to extract the mean grey values of single beads.

| Sample | Exposure time | Mean grey value single beads |
|---|---|---|
| Colored porous bead | 100 ms | 1038 |
| Porous bead blank reference | 100 ms | Not detectable |

**Example 3: Colored magnetisable monosized beads**

4,5 $\mu$M porous magnetisable beads were made according to WO 00/68692 and patent no EP 0106873.

Coating with butyldiglycidylether

7 gram magnetisable beads from above were dispersed in 35 gram butyldiglycidylether. The mixture was heated to 80$^0$C. After 4 hours the dispersion was cooled down to room temperature and repeatedly washed with toluene and then acetone. The reaction gave 8.6 gram magnetisable coated beads.

Modification with 1,6 diaminohexane

6.5 gram of the beads above were dispersed in 110 gram butylacetate. 13 gram of 1.6 diaminohexane was added to the dispersion. The mixture was heated to 70$^0$C and left for 2 hours at 70$^0$C with shaking. The dispersion was washed sequentially with a 50wt% mixture of butylacetate and methanol until all the excess of amine was removed (confirmed by ninhydrine-test). The beads were

dispersed in toluene to a final dry content of 11 wt%.

Adsorption of TOP/TOPO Qdot to the bead matrix.

50 $\mu$L of a 2.5 $\mu$M dispersion of TOP/ TOPO Qdot (Quantum Dot Corp.) with an emission maximum at 608 nm were flocculated by adding 50 $\mu$L methanol. The supernatant was removed and the quantum dot precipitate were added 50 $\mu$l toluene and further 200 $\mu$L of a 1:1 mixture of toluene and 1,4 dioxane.

50 mg of the above beads were dispersed in a 1:1 mixture of toluene and 1,4 dioxane to total weight of 1 gram.

The Qdot dispersion was added the bead dispersion while vortexing. After 15 hours the beads were washed with 1,4 dioxane.

A sample was taken out and transferred to water.

Fluorescence microscopy showed that the beads were fluorescent:

| Sample | Exposure time | Mean gray value single beads |
|---|---|---|
| Magnetisable colored bead | 300 ms | 861 |
| Magnetisable bead blank reference | 300 ms | Not detectable |

Cross-linking reaction

To the magnetisable colored beads in 1,4 dioxane (5 wt%) is added 5 mg isophorone diisocyanate and the mixture

is heated to 60°C for 3 hours.
The beads are transferred to water. Fluorescence microscopy shows that the beads are fluorescent.

**Example 4: Colored compact monosized beads**

Compact epoxy-functional acrylic beads with a cross linker content of 5 wt% ethylendimethacrylate were made according to WO 00/68692 and US 4,336,173.

Amination

To 300 g of a compact bead dispersion in water (dry-content 9,60 wt%) 14.10 g of 3-amino-1,2-propanediol was added. The suspension was heated to 80°C and stirred for 18 hours. After cooling the particles were washed with water, methanol and butyl acetate. The final dry-content was 4.9%.

Staining

1 mL of 1.9 $\mu$M TOP/TOPO Qdot dispersion with an emission maximum at 625 nm was added 2.2 mL butyl acetate. 12.5 mL of the above bead dispersion was mixed with the Qdot dispersion. 23,0 mL 1,4-butyldiglycidylether was slowly added while stirring. One drop of $BF_3$-ether complex was added. The mixture was heated to 75°C. After 20 hours the dispersion was cooled and washed with butyl acetate and isopropanol. The particles were finally dispersed in water containing 1,5 g/L sodiumdodecylsulphate.

The beads were analyzed with the fluorescence microscope with a long pass emission filter with transmission of wavelengths above 520 nm.

| Sample | Exposure time | Mean grey value |
|---|---|---|
| | | Single beads |
| Compact colored bead | 50 | 2578 |
| Compact bead | 50 | Not detectable |

Claims

1. A probe comprising an optionally porous, polymer bead with at least one type of nanocrystals associated therewith, said bead and nanocrystals being coated with a polymeric coating.

2. A probe as claimed in claim 1 wherein said probe comprises at least two different types of nanocrystals.

3. A probe as claimed in claim 1 or 2 wherein said nanocrystals are between 2 to 20 nm in diameter.

4. A probe as claimed in any one of claims 1 to 3 wherein said nanocrystals are essentially spherical and monodisperse.

5. A probe as claimed in any one of claims 1 to 4 wherein said nanocrystals are made from CdS, CdSe, CdTe, ZnSe, ZnTe, GaP or GaAs.

6. A probe as claimed in any one of claims 1 to 5 wherein said nanocrystals are coated with an organic capping agent which is hydrophilic, amphiphilic or hydrophobic.

7. A probe as claimed in claim 6 wherein said capping agent is of formula $O=P(C_{6-12}\text{-alkyl})_3$ or $P(C_{6-12}\text{-alkyl})_3$.

8. A probe as claimed in any one of claims 1 to 7 wherein said bead is a Dynosphere-R™.

9. A probe as claimed in any one of claims 1 to 8 wherein the polymeric coating comprises an epoxy or polyurethane polymer.

10. A probe as claimed in any one of claims 1 to 9

wherein said nanocrystals are deposited on the bead surface.

11. A probe as claimed in any one of claims 1 to 10 wherein said bead surface carries functional groups.

12. A probe as claimed in claim 11 wherein said functional groups comprise amines, thiols, phosphines, phosphine oxides, pyridine or furans.

13. A probe as claimed in any one of claims 1 to 12 wherein said beads are aminated.

14. A probe as claimed in any one of claims 1 to 13 wherein said bead is substantially non-porous.

15. A probe as claimed in any one of claims 1 to 13 wherein said bead is porous.

16. A probe as claimed in claim 15 wherein said nanocrystals are present in the bead pores.

17. A probe as claimed in any one of claims 1 to 16 wherein said bead is magnetisable.

18. A probe as claimed in claim 17 wherein said bead is a Dynabead-R™.

19. A probe as claimed in claim 17 or 18 wherein the bead surface is nitrated.

20. A probe as claimed in any one of claims 15 to 19 wherein said bead is magnetisable and porous, magnetisable particles being present within the pores of the bead.

21. A probe as claimed in any one of claims 1 to 20

wherein said polymeric coating comprises at least one affinity ligand.

22. A probe as claimed in claim 21 wherein said affinity ligand is selected from monoclonal antibodies, polyclonal antibodies, antibody fragments, nucleic acids, oligonucleotides, proteins, polysaccharides, sugars, peptides, peptide nucleic acid molecules, antigens, drugs and ligands.

23. A probe as claimed in claim 1 comprising a porous bead, magnetisable particles being present in the pores thereof, a first coating on said bead, nanocrystals deposited on said first coating and a second coating over said nanocrystals and bead.

24. A probe as claimed in claim 23 wherein said second coating is formed by cross-linking said first coating.

25. A probe as claimed in claim 24 wherein said first coating is an uncrosslinked epoxy polymer and said second coating is an epoxy polymer cross-linked with a diisocyanate, diamine or epoxide.

26. A plurality of probes as claimed in any one of claims 1 to 25 wherein said beads are monodisperse and have a particle size distribution of less than 5%.

27. Probes as claimed in claim 26 wherein magnetisable particles are distributed within the bead.

28. Probes as claimed in claim 27 wherein magnetisable particles are evenly distributed within the bead.

29. Use of probes as claimed in any one of claims 26 to 28 in a concentration dependent multiplex assay.

30. Use of a probe as claimed in any one of claims 1 to 25 in an assay.

31. Use of a probe as claimed in any one of claims 1 to 25 in an assay for the detection of nucleic acid, protein, cells, microorganisms, viruses or in an assay for the diagnosis of a disease.

32. Use of a probe as claimed in any one of claims 1 to 25 as a detectable reporter or solid phase in a microfluidic device.

33. A process for the preparation of a luminescent probe comprising mixing an optionally porous, polymer bead with at least one type of nanocrystals so that the nanocrystals associate with said bead and subsequently coating said bead to form said probe.

34. A process as claimed in claim 33 wherein said polymer bead has a coating comprising functional groups which coordinate with the shell of the nanocrystals.

35. A process as claimed in claim 34 wherein said functional groups are diamines, thiols, phosphines, phosphine oxides, pyridines or furans.

36. A process as claimed in claim 35 wherein said polymer beads are aminated.

37. A method for detecting an analyte in a sample comprising mixing said sample with at least one probe as claimed in any one of claims 1 to 25 said probe carrying at least one affinity ligand; allowing the analyte to bind to said probe; and detecting the resulting probe-analyte conjugate.

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(54) Title: POLYMERIC BEAD PROBE WITH NANOCRYSTAL, MANUFACTURE AND USE OF THE SAME

(57) Abstract: A probe comprising an optionally porous, polymer bead with at least one type of nanocrystals associated therewith, said bead and nanocrystals being coated with a polymeric coating.

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# INTERNATIONAL SEARCH REPORT

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 00 68692 A (DANIELS R HUGH ;WONG EDITH Y (US); BRUCHEZ MARCEL P (US); EMPEDOCL) 16 November 2000 (2000-11-16) cited in the application the whole document | 1-37 |
| X | CHAN WCW ET AL: "Quantum Dot bioconjugates for ultrasensitive nonisotopic detection" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 281, 25 September 1998 (1998-09-25), pages 2016-2018, XP002125871 ISSN: 0036-8075 the whole document | 1-37 |

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Int nal Application No
PCT/GB 02/02590

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 00 28088 A (BIOCRYSTAL LTD) 18 May 2000 (2000-05-18) the whole document | 1-37 |
| X | WO 00 27365 A (BIOCRYSTAL LTD) 18 May 2000 (2000-05-18) the whole document | 1-37 |
| X | WO 00 17656 A (MASSACHUSETTS INST TECHNOLOGY) 30 March 2000 (2000-03-30) the whole document | 1-37 |
| X | WO 00 58731 A (COULTER INT CORP) 5 October 2000 (2000-10-05) the whole document | 1-37 |
| X | WO 00 17103 A (MASSACHUSETTS INST TECHNOLOGY ;JENSEN KLAVS F (US); BAWENDI MOUNGI) 30 March 2000 (2000-03-30) the whole document | 1-37 |
| X | WO 00 68669 A (MASSACHUSETTS INST TECHNOLOGY) 16 November 2000 (2000-11-16) the whole document | 1-37 |

# INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/GB 02/02590

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 0068692 A | 16-11-2000 | AU 4701200 A | 21-11-2000 |
| | | EP 1179185 A1 | 13-02-2002 |
| | | JP 2002544488 T | 24-12-2002 |
| | | WO 0068692 A1 | 16-11-2000 |
| | | US 6274323 B1 | 14-08-2001 |
| | | US 2001055764 A1 | 27-12-2001 |
| | | US 2001034034 A1 | 25-10-2001 |
| WO 0028088 A | 18-05-2000 | US 6261779 B1 | 17-07-2001 |
| | | AU 1616100 A | 29-05-2000 |
| | | AU 1717500 A | 29-05-2000 |
| | | AU 1911700 A | 29-05-2000 |
| | | EP 1157133 A1 | 28-11-2001 |
| | | WO 0027436 A1 | 18-05-2000 |
| | | WO 0028088 A1 | 18-05-2000 |
| | | WO 0028089 A1 | 18-05-2000 |
| | | US 2002150905 A1 | 17-10-2002 |
| | | AU 2147000 A | 29-05-2000 |
| WO 0027365 A | 18-05-2000 | US 6114038 A | 05-09-2000 |
| | | AU 1616100 A | 29-05-2000 |
| | | AU 2147000 A | 29-05-2000 |
| | | EP 1128818 A1 | 05-09-2001 |
| | | WO 0027365 A1 | 18-05-2000 |
| | | WO 0028089 A1 | 18-05-2000 |
| | | US 6221602 B1 | 24-04-2001 |
| | | AU 1911700 A | 29-05-2000 |
| | | WO 0027436 A1 | 18-05-2000 |
| | | US 6333110 B1 | 25-12-2001 |
| | | US 6309701 B1 | 30-10-2001 |
| | | US 6319607 B1 | 20-11-2001 |
| WO 0017656 A | 30-03-2000 | US 6426513 B1 | 30-07-2002 |
| | | WO 0017656 A1 | 30-03-2000 |
| WO 0058731 A | 05-10-2000 | US 6235540 B1 | 22-05-2001 |
| | | EP 1166120 A1 | 02-01-2002 |
| | | JP 2002540428 T | 26-11-2002 |
| | | WO 0058731 A1 | 05-10-2000 |
| | | US 2001039060 A1 | 08-11-2001 |
| WO 0017103 A | 30-03-2000 | US 6251303 B1 | 26-06-2001 |
| | | US 6326144 B1 | 04-12-2001 |
| | | US 6306610 B1 | 23-10-2001 |
| | | US 6319426 B1 | 20-11-2001 |
| | | AU 1092300 A | 10-04-2000 |
| | | AU 6392399 A | 10-04-2000 |
| | | CA 2344145 A1 | 30-03-2000 |
| | | CA 2344478 A1 | 30-03-2000 |
| | | EP 1116036 A1 | 18-07-2001 |
| | | EP 1113986 A2 | 11-07-2001 |
| | | JP 2002525394 T | 13-08-2002 |
| | | WO 0017103 A2 | 30-03-2000 |
| | | WO 0017655 A1 | 30-03-2000 |
| | | WO 0017642 A2 | 30-03-2000 |
| | | US 2002160412 A1 | 31-10-2002 |
| | | EP 1271154 A2 | 02-01-2003 |
| | | EP 0990903 A1 | 05-04-2000 |

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/02590

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 0017103 A | | GB 2342651 A ,B | 19-04-2000 |
| | | US 2001023078 A1 | 20-09-2001 |
| | | AU 6148599 A | 10-04-2000 |
| | | CA 2344479 A1 | 30-03-2000 |
| | | US 2001040232 A1 | 15-11-2001 |
| WO 0068669 A | 16-11-2000 | WO 0068669 A1 | 16-11-2000 |